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Efficacy of β -resorcylic Acid to Reduce *Campylobacter jejuni* in Pre-harvest and Post-harvest
Poultry

Efficacy of β -resorcylic Acid to Reduce *Campylobacter jejuni* in Pre-harvest and Post-harvest
Poultry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Poultry Science

By

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Tribhuvan University
Bachelor of Veterinary Science & Animal Husbandry, 2010

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ABSTRACT

Campylobacteriosis is one of the leading foodborne illnesses in United States, and is associated with the consumption of poultry and poultry products. Reducing *Campylobacter* in these species will reduce the burden of this disease. Unfortunately, most strategies employed to reduce *Campylobacter* in poultry have either not been successful or produced inconsistent results. One potential control strategy is the use of β -resorcylic acid (BR), a phytophenolic compound classified by the US FDA as “Everything Added to Food in the United States” (EAF 3045) and is therefore deemed safe for consumption. This compounds has antibacterial activity against *Salmonella*, however, its efficacy to control *Campylobacter* in poultry has not been evaluated. Preliminary studies in our laboratory demonstrated that BR kills *Campylobacter jejuni*, *in vitro*. Therefore, the objective of this study was to evaluate if BR would reduce *Campylobacter* in chickens. In pre-harvest studies, day of hatch chicks were fed one of five treatments (0, 0.25, 0.5, 1 or 2%BR) in the first trial, whereas a second trial was conducted including two additional doses of 0.75% & 1.5% BR (n=10 chicks/dose). Birds were challenged with mixture of four wild strains of *C. jejuni* ($\sim 10^6$ CFU/mL) on day 7 and cecal samples were collected on day 14 and enumerated for *Campylobacter*. In post-harvest studies, four trials, two each on thigh skin and breast meat, were conducted. Chicken skin or meat samples (2 ± 0.1 g) were inoculated with 50 μ L of *C. jejuni* ($\sim 10^7$ CFU/mL). Following 30 min of attachment, samples were dipped into their respective treatment solutions (0, 0.5, 1, 2% BR) for 30 s and suspended 2 min (n=10 samples/dose) and evaluated for reduction in *Campylobacter* counts. *Campylobacter* counts were reduced by 1.4 Log CFU/g for the 2% dose in the first pre-harvest trial and by 4.2 or 2.8 Log CFU/g for the 0.5 % & 1% BR doses in the second pre-harvest trial (P<0.05). In the post-harvest studies, all doses of BR significantly reduced *Campylobacter*

counts in both meat and skin. Results of these experiments suggest post-harvest application of BR is the most effective treatment and may help reduce the incidence of human campylobacteriosis.

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DEDICATION

I would like to dedicate this thesis work to my father, Bhoj Raj Wagle and my mother, Nanda Kumari Wagle for their unwavering love, support and instilling in me an energy and work ethic to accomplish my career goals.

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CHAPTER 1:

INTRODUCTION

Foodborne diseases are a growing public health concern and *Campylobacter* is one of the leading causes of bacterial foodborne illnesses (WHO, 2012; CDC, 2014). The Centers for Disease Control and Prevention (CDC) estimated the incidence of campylobacteriosis to be 13.83 per 100,000 humans with a loss of \$ 1.7 billion per annum (Hoffmann, et al., 2012; CDC, 2014). In the European Union, approximately 9 million cases annually of human campylobacteriosis are estimated resulting in a € 2.4 billion economic cost (EFSA Panel on Biological Hazards, 2011). *Campylobacter* infections in humans is also implicated in causing Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome (Berden, et al., 1979; Rhodes and Tattersfield, 1982; Smith, 2002; Pope, et al., 2007; Gradel, et al., 2009). Risk assessment studies on *Campylobacter* identified poultry and poultry products as a major source of human infections (Rosenquist, et al., 2003; Friedman, et al., 2004; FAO/WHO, 2009). Source attribution studies indicated handling of raw chicken, consumption of undercooked meat and cross-contamination during food preparation are likely causes for human *Campylobacter* infections (Hopkins and Scott, 1983; Altekruze, et al., 1999; Friedman, et al., 2004; Danis, et al., 2009; Lindmark, et al., 2009; Fajo-Pascual, et al., 2010). *Campylobacter jejuni* has been reported as the most common species causing campylobacteriosis (Friedman, 2000; FAO/WHO, 2009) and is the most common species found in poultry (Suzuki and Yamamoto, 2009). It has been estimated that a reduction of *Campylobacter* counts by 2 Log on chicken carcasses will reduce the incidence of this disease causing bacterium in humans by 30 fold (Rosenquist, et al., 2003). Therefore, the aim of this thesis research is to reduce *Campylobacter* in both pre-harvest and post-harvest poultry by using the novel compound, β -resorcylic acid.

HISTORICAL PERSPECTIVE

A non-culturable and spiral bacteria was first observed by Theodore Escherich in the colon of children who died from cholera infantum (Escherich, 1886). The first isolation of a *Vibrio*-like organism from ovine aborted fetuses was accomplished by two veterinary surgeons, McFadyean and Stockman in 1913 (Butzler, 2004; Skirrow, 2006; Debruyne, et al., 2008). Smith and Taylor (1919) found similar organisms associated with diseased fetal membranes in cattle and proposed the name *Vibrio fetus*. The term, *Vibrio jejuni* (Jones and Little, 1931) and *Vibrio coli* (Doyle, 1944) were given to a group of similar bacteria causing winter dysentery in calves and swine dysentery, respectively (Jones, et al., 1931; Doyle, 1944). In 1946, Levy noted the presence of spiral organisms microscopically in the blood of people with gastrointestinal disease but was unable to recover by culture (Levy, 1946). Vincent and colleagues (1947) successfully isolated *Vibrio fetus* from the blood of a pregnant woman suffering from fever of unknown origin in the following year. King first studied the strains of *Vibrio* and described a new strain with different antigenic and biochemical characteristics but several common features with the agent reported by Vincent (King, 1957; King, 1962). In 1963, Sebald and Véron proposed the new genus *Campylobacter* separating them from true *Vibrio* due to their low DNA base composition, microaerophilic growth requirements and nonfermentative metabolism (Sebald and Véron, 1963). Isolation of *Campylobacter* from human feces was first accomplished in 1968 and the significance of genus members as human enteric pathogens was elucidated (Dekeyser, et al., 1972; Skirrow, 1977). Additional organisms identified in 1973 as *Vibrio*-like microaerophilic bacteria were classified under the same genus *Campylobacter* including *Vibrio jejuni* and *Vibrio coli* (Butzler, 2004). The genus *Campylobacter* later was incorporated into a new family, *Campylobacteraceae*, along with other genera; *Helicobacter*, *Arcobacter*, *Sulfurospirillum*, and

Wolinella (Debruyne, et al., 2008). *Campylobacter* species are the most common bacterial cause of diarrhea worldwide as was determined by the mid to end 1980s (Allos, 2001). Currently, there are 17 species of *Campylobacter* under the genus *Campylobacter* (Lastovica, 2006; Debruyne, et al., 2008). *Campylobacter jejuni* and *Campylobacter coli* account for over 95% of the human *Campylobacter* infections (Park, 2002; Snelling, et al., 2005).

CHARACTERISTICS OF CAMPYLOBACTER

Morphological characteristics

The word *Campylobacter* is made of up two Greek words, “kampulos” meaning “curved” and “bacter” meaning “rod” (Sebald and Véron, 1963). Members of the genus *Campylobacter* are gram negative, non-spore forming, mostly slender, spirally curved rods 0.2 to 0.8µm wide and 0.5 to 5µm long (Thomas, et al., 1998; Debruyne, et al., 2008). This bacterium can also transform into a coccoid viable but nonculturable (VBNC) state under unfavorable conditions (Rollins and Colwell, 1986). Most species are motile with characteristic cork-screw-like motion by means of long, single, polar flagellum two to three times the length of cells (Smibert, 1978; Debruyne, et al., 2008). Some exceptions are *C. gracilis*, which is nonmotile, and *C. showae*, which have multiple flagella (Debruyne, et al., 2008).

Growth requirements

Most of the *Campylobacter* spp are nutritionally fastidious and require a microaerophilic environment for growth (Kelly, 2001; Park, 2002; Garénaux, et al., 2008). Some species are almost anaerobic, however, the majority are microaerophilic growing optimally in the presence of 5-10% oxygen (Thomas, et al., 1998; Park, 2002). They need a complex media with additional growth supplements for culture (Buck and Smith, 1987; Kelly, 2001). Furthermore, thermophilic

Campylobacters have restricted growth temperature requirements, optimally growing at 42°C and not below 30°C (Thomas, et al., 1998; Park, 2002; Jackson, et al., 2009). However, *C. jejuni* displays physiological activity even at 4°C (Hazeleger, et al., 1998). *Campylobacter jejuni* undergoes a morphological change from spiral to coccoid form after several days of growth, *in vitro* (Kelly, 2001). Unfortunately, conditions like stress, temperature, pH, osmolarity, and medium are responsible for transition from spiral to coccoid VBNC state (Rollins and Colwell, 1986; Lázaro, et al., 1999; Moore, 2001; Jackson, et al., 2009). The VBNC state, first described by Rollins and Colwell (1986), is a survival mechanism under unfavorable conditions in which the ability to culture is lost even though the microorganism is alive and metabolically active (Oliver, 2005; Svensson, et al., 2008). *Campylobacter jejuni* may enter the VBNC state due to abiotic stress making their detection difficult during quality control testing via culture methods and therefore creating a challenge for the food processing industry (Jackson, et al., 2009). Some studies have reported that these VBNC cells may still retain their ability to culture and to colonize or infect the host (Saha, et al., 1991; Cappelier, et al., 1999; Rowan, 2004; Baffone, et al., 2006) but not in other studies (Ziprin, et al., 1999; Hald, et al., 2001; Ziprin, et al., 2003).

CAMPYLOBACTER IN HUMANS

Sources of infection

The reservoirs of thermophilic *Campylobacter* responsible for human infections comprises food production animals like chicken, turkey, ducks, cattle, sheep and pigs (Penner, 1988; Skirrow, 1982; Blaser, 1997; Stanley and Jones, 2003; Stojanov, et al., 2004; Humphrey, et al., 2007) wild birds and reptiles (Luechtefeld, et al., 1980; Kapperud and Rosef, 1983; Harvey and Greenwood, 1985; Pacha, et al., 1987; Pacha, et al., 1988; Tresierra-Ayala and Fernandez, 1997). Water sources (e.g., ponds) and protozoan also can serve as reservoirs (Jones, 2001;

Axelsson-Olsson, et al., 2005). Chicken as a primary source of infection was mentioned by King (King, 1962; Skirrow, 1977) and presently constitute by far the largest potential source of human infections (Butzler, 2004). The handling and consumption of poultry are a major source of infections as reported and accepted worldwide in many studies (Hopkins and Scott, 1983; Ikram, et al., 1994; Altekruse, et al., 1999; Neimann, et al., 2003; Friedman, et al., 2004; Stafford, et al., 2008; Danis, et al., 2009; Lindmark, et al., 2009; Fajo-Pascual, et al., 2010). Cross-contamination during food preparation is also significant risk factor (Boer and Hahne, 1990; Mylius, et al., 2007; Lubert, 2009). Other sources are consumption of raw or unpasteurized milk (Porter and Reid, 1980; Robinson and Jones, 1981; Taylor, et al., 1982; Potter, et al., 1983; Kornblatt, et al., 1985; Peterson, 2003), fruits and vegetables (Evans, et al., 2003; Verhoeff-Bakkenes, et al., 2011), untreated water (Vogt, et al., 1982; Palmer, et al., 1983; Taylor, et al., 1983; Hopkins, et al., 1984; Sacks, et al., 1986; Melby, et al., 1991), mushrooms (Doyle and Schoeni, 1986) as well as direct transmission of *Campylobacter* from animals to humans (e.g., dogs; Rosef and Kapperud, 1983). Another possible route of infection is direct contact with domestic animals and pets (Deming, et al., 1987; Kapperud, et al., 1992; Studahl and Andersson, 2000; Workman, et al., 2005).

Human incidence

With the development of a selective medium by Skirrow (1977) and methods by Dekeyser and coworkers (1972) to identify and isolate *Campylobacter* from human samples, a more accurate assessment of the disease burden is possible. Campylobacteriosis remains an emerging foodborne disease globally since 1980s (WHO, 2012; EFSA, 2013; CDC, 2014). In the United States, the CDC estimated an incidence of 13.83/100,000 humans in 2013 which is significantly higher than data reported in 2006-2008 (CDC, 2014). However, true incidence is

expected to be even higher because many cases are undiagnosed or underreported (Mead, et al., 1999; Samuel, et al., 2004). In the European Union, campylobacteriosis was the most commonly reported zoonotic disease followed by salmonellosis in 2011 (EFSA/ECDC., 2013). The European Food Safety Authority and the European Centre for Disease Control and Prevention jointly reported 220,209 *Campylobacter* cases in 2011, 2.2% more than in 2010 (EFSA, 2013). Annual cases of 225,000 were estimated in Australia and 500,000 in England and Wales, (Hall, et al., 2008; Nichols, et al., 2012). Increasing incidences were also reported from the Netherlands (Havelaar, et al., 2012), Germany (Schielke, et al., 2014), Finland (Nakari, et al., 2010) and Israel (Weinberger, et al., 2013).

The incidence of human campylobacteriosis has shown variation in regard to age, sex and season (Friedman, 2000; Nelson and Harris, 2011). The highest rates of cases were reported in infants and young adults in developed countries (Galanis, 2007; Schielke, et al., 2014). The incidence was 24.08 per 100,000 in infants (<5year) followed by 14.54 per 100,000 in the 20-64 age group in the United States (CDC, 2014). Rates of *Campylobacter* infection are, in general, higher in males with approximate male to female ratio of 1.2 to 1 (Louis, et al., 2005; Olson, et al., 2008). The reason behind this inequality was not fully explained (Friedman, 2000; Allos, 2001). Studies in seasonality index for campylobacteriosis have stated a peaked incidence during June-August in North America, Europe, UK and Canada (Nylen, et al., 2002; Nelson and Harris, 2011; Lal, et al., 2012). Seasonal distribution of *Campylobacter* incidence may correspond with the increased prevalence of *Campylobacter* in reservoirs and sources and also the seasonal variation in human behavior which exposes people to *Campylobacters* (Nylen, et al., 2002). In developing countries, the number of cases campylobacteriosis is unknown due to a lack of national surveillance programs (Coker, et al., 2002).

Pathogenesis of campylobacteriosis

Even though there is significant knowledge about the clinical, microbiological and epidemiological aspects of *Campylobacter* infections, the molecular mechanisms involved in pathogenesis are still poorly understood (Ketley, 1997). The initiation and establishment of *C. jejuni* infection are thought to involve adherence, protein secretion, and invasion which can stimulate the host cell inflammatory response (Larson, et al., 2008). Its bacterial flagellum aids in infection by facilitating motility as well as secretion of *Campylobacter* invasion antigen (Dasti, et al., 2010). Binding of *Campylobacter* to a host cell is mediated by two fibronectin binding proteins, CadF and FlpA (Konkel, et al., 1997; Konkel, et al., 2010). Then, *Campylobacter* invasion antigen modify the host cell regulatory pathway and promote host cell invasion and intracellular survival (Konkel, et al., 1999; Konkel, et al., 2004). It has been reported that microtubules and microfilaments of host cells trigger the internalization of *Campylobacter* (Biswas, et al., 2003). Cytolethal distending toxins (Cdt A, B, C), major pathogenicity-associated factors, have been recognized recently that play a key role in arresting the cell cycle and inducing host cell apoptosis (Dasti, et al., 2010).

Human infection

Human *Campylobacter* infection is one of the most frequent causes of gastroenteritis in the United States and the world (Blaser, et al., 1983; WHO, 2012). It is more commonly reported in children, those immunocompromised and elderly people (Allos, 2001; Corry and Atabay, 2001). Infection is regarded as a foodborne disease rather than food poisoning and the majority of infections are sporadic (Griffiths and Park, 1990; Friedman, et al., 2004). Thermophilic *Campylobacters* namely *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* are responsible for campylobacteriosis (Thomas, et al., 1998). However, *C. jejuni* is the most commonly reported

followed by *C. coli*, together contributing over 95% of the total infections globally (Park, 2002; EFSA/ECDC., 2013). *Campylobacter coli* is more sensitive to antibiotics used in selective media and less survivable in colder temperature which may lead to an underestimate of its incidence in comparison to *C. jejuni* (Griffiths and Park, 1990). Studies on infective dose of *C. jejuni* have determined a very low infective dose of 500-800 cells (Robinson, 1981; Black, et al., 1988). *Campylobacter jejuni* is known to cause self-limiting illnesses with initial symptoms usually seen 2-5 days after oral ingestion but sometimes extending up to 10 days and characterized by onset of diarrhea, abdominal cramps and preceded by fever (Skirrow, 1977; Butzler, 2004). It has been noted that symptoms begin with mild watery diarrhea which soon becomes bloody. Bloody diarrhea is regarded as inflammation due to invasive nature of *C. jejuni* leading to enteritis and colitis (Russell, et al., 1993; Blaser, 1997). However, extra intestinal manifestations like meningitis, osteomyelitis and neonatal sepsis have been reported, although rarely (Butzler, 2004). Various complications such as reactive arthritis, Guillian-Barré syndrome, irritable bowel syndrome and inflammatory bowel disorder may occur as sequelae of *Campylobacter* infection (Gumpel, et al., 1981; Allos, 1997; Hannu, et al., 2002; Yuki, et al., 2004; Spiller and Garsed, 2009).

Guillian-Barré syndrome. Guillian-Barré syndrome (GBS) is a post-infectious auto-immune mediated neuropathy with characteristic rapid progressive weakness, sensory loss and slow clinical recovery (van Koningsveld, et al., 2007). *Campylobacter* infection has been known to precede GBS and their association was reported as early as 1982 by Rhodes and Tattersfield (Rhodes and Tattersfield, 1982). *Campylobacter jejuni* is the most frequently observed antecedent infection in cases of GBS and symptoms usually noted 1-3 weeks after onset of *Campylobacter* enteritis (Butzler, 2004). It is the most serious complication of

campylobacteriosis and approximately 20% of the patients are admitted to the hospital in the intensive care unit for respiratory ventilation (WHO, 2012). Guillian-Barré syndrome is reported to occur in 1 out of every 1000 campylobacteriosis cases (Allos, 1997). Serological studies have estimated that 20-40 % of GBS patients had *C. jejuni* infection before the onset of GBS (Mishu and Blaser, 1993). Guillian-Barré syndrome can occur at any age but occurs more frequently with increasing age and is slightly more common in males than females with a ratio of 1.25 (Hadden and Gregson, 2001).

Four subtypes of GBS are acute inflammatory demyelinating polyadiculoneuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor and sensory axonal neuropathy (AMSAN), and Fisher's syndrome. Antecedent of *Campylobacter* infection is detected more frequently in AMAN subtype (Kuwabara, 2004). Chemical characterization of *C. jejuni* lipopolysaccharides indicates the presence of sialic acid and further investigation by Yuki and associates (1993) revealed an identical structure between the terminal structure of oligosaccharide of *C. jejuni* Penner's serotype 19 and terminal tetrasacchharide of GM₁ ganglioside, establishing a molecular mimicry between them (Moran, et al., 1991; Yuki, et al., 1993; Yuki, et al., 2004). Following infection with *Campylobacter*, the immune system is suspected of producing anti-ganglioside-reactive antibodies that cause antibody-complement-mediated demyelination and/or axonal damage resulting in symptoms of GBS (Nachamkin, 2001).

Recovery from GBS is variable and fatality rates ranges from 2 to 18%, usually resulting from cardiovascular autonomic complications, ventilator-associated pneumonia and pulmonary thromboembolism (Hadden and Gregson, 2001). *Campylobacter jejuni* is also found in association with Fisher's syndrome. Infection with *Campylobacter* produces the antibodies

against the GQ_{1b} ganglioside, which damages the ocular motor nerve (Hughes and Cornblath, 2005). As a consequence, unique clinical signs like ophthalmoplegia (paralysis of eye muscle), ataxia (lack of coordinated movement of muscle) and areflexia (absence of nerve reflexes) are commonly seen (Kuwabara, 2004).

Reactive arthritis. Reactive arthritis, a spondyloarthropatic disorder, is characterized by inflammation of joints and tissues occurring after gastrointestinal or genitourinary infection (Ajene, et al., 2013). The association of reactive arthritis with *C. jejuni* was first reported by Berden in 1978 (Berden, et al., 1979). It develops within 2 weeks after *Campylobacter* related gastroenteritis in humans (Berden, et al., 1979). The incidence of reactive arthritis varies from 0 to 16% with a mean of 0.9% in *Campylobacter* infections (Ajene, et al., 2013). However, it is difficult to get true incidence because of varied clinical severity and milder cases that go frequently unreported (Pope, et al., 2007). Even though the burden of campylobacteriosis is higher among children < 5years, estimates indicate lower incidence of reactive arthritis in that particular age group (Ajene, et al., 2013; CDC, 2014). The most commonly affected age group is young adults, and both sexes have an equal chance of acquiring this illness (Pope, et al., 2007). The proposed pathogenesis is that bacteria, following invasion of mucosa, persist in the epithelium associated lymph node, liver and spleen which then disseminate into the joints causing inflammation and are often supported by CD₄⁺ T-cells (Hill Gaston and Lillicrap, 2003).

Irritable bowel syndrome. Irritable bowel syndrome (IBS) is a relapsing functional disorder characterized by abdominal pain or discomfort associated with defecation or change in bowel habits (Quigley, et al., 2009). This illness is characterized by 2 or more of the following; fever, vomiting, acute diarrhea, or a positive bacteria stool culture (Spiller, 2007). The

prevalence of IBS in North America and Europe ranges 10-15% (Quigley, et al., 2009). About 10% of IBS cases are reported to be caused by *Campylobacter* spp (Spiller and Garsed, 2009). The mechanism by which IBS develops with *Campylobacter* infection is not fully understood. It may be due to the proteins secreted by the organisms which cause bowel disturbances (Thornley, et al., 2001).

Inflammatory bowel disorder. Inflammatory bowel disorder (IBD) is a poorly understood chronic disorder of the intestine and includes ulcerative colitis and Crohn's disease with an incidence of 2 to 4 per 100,000 population (Papadakis and Targan, 1999; Bernstein, 2009). *Campylobacter jejuni* has also been associated with the inflammatory bowel disorder and has been isolated from 10% of the IBD cases (Gradel, et al., 2009; WHO, 2012). The suggested mechanism is that pathogenic bacteria like *C. jejuni* causes cellular damage, either by cytotoxic effect and/or by host cell invasion, which promotes translocation of commensal bacteria and activation of T-cells leading to inflammation (Kalischuk, et al., 2009).

Treatment and economic burden

Even though milder cases of campylobacteriosis do not need antibiotic treatment, in circumstances such as high fever, prolonged illness (>1 weeks), bloody diarrhea, infection in pregnancy, HIV and immunocompromised individuals require treatment (Allos and Blaser, 1995; Allos, 2001). At one time, fluoroquinolones, specifically ciprofloxacin was used as the drug of choice (Allos and Blaser, 1995). The consequence of this therapy resulted in increased fluoroquinolone resistant *Campylobacter* strains in many parts of the world (Gaunt and Piddock, 1996; Allos, 2001). The first report for the United States showed an increase of quinolone resistant *Campylobacter* isolates from 1.3 to 10.2% during the years 1992-1998 (Smith, et al.,

1999). Another study from 2002 to 2007 determined an increase from 15.2 to 17.2% of ciprofloxacin resistant *C. jejuni* in the United States (Zhao, et al., 2010). Presently, erythromycin is becoming popular as the treatment of choice due to its low toxicity, narrow spectrum activity, and low cost (Allos and Blaser, 1995). However, overall cost for treatment of campylobacteriosis is often expensive. In the United States, estimates have shown an approximate cost of \$1846 per treatment for each case of campylobacteriosis with a total economic burden of approximately \$1.7 billion per annum (Hoffmann, et al., 2012; Scharff, 2012). Similarly, the cost of illness per case in New Zealand was \$600, in total contributing 90% of the total cost (\$86million) of foodborne illness (Lake, et al., 2010). A report from European Union estimated an economic loss of €2.4 billion from campylobacteriosis (EFSA Panel on Biological Hazards, 2011). These burden studies highlight the need of control measures through multistep intervention to reduce the load of *Campylobacter* on the farm as well as in the processing facility (WHO, 2012).

CAMPYLOBACTER IN POULTRY

Incidence in poultry

Campylobacter is widely present in poultry worldwide. Studies have reported *Campylobacter* incidence up to 90% of poultry flocks in the United States (Stern, et al., 2001), 47.5% in Japan (Haruna, et al., 2012), 41.1% in Germany (Atanassova and Ring, 1999), 50% in organic chickens in Quebec (Thibodeau, et al., 2011) and more than 90% in Great Britain (Evans and Sayers, 2000). The incidence in European countries varies from 2% to 100% with an average of 71.2% in broiler flocks (EFSA, 2010). Higher incidences were reported from Luxemburg (100%), Malta (96.8%) and Spain (88%) (EFSA, 2010). The incidence of *Campylobacter* in poultry meat and its products is variable. One study determined 70.7% of broiler carcasses in supermarkets were contaminated in Washington, D.C. (Zhao, et al., 2001). Rates of

Campylobacter in broiler carcasses were 45.9% in Germany (Atanassova and Ring, 1999), 93.2% in Australia (Pointon, et al., 2008), 64.7% in Japan (Sallam, 2007), 59.3% in Iran (Zendehbad, et al., 2013), 20.8% in Estonia (Mäesaar, et al., 2014), 84.3% in the Republic of Ireland (Madden, et al., 2011) and 91% in Northern Ireland, Scotland, England and Wales (Moran, et al., 2009). Overall EU prevalence of *Campylobacter* in broiler meat is 75.8 % (EFSA, 2010). This variability in prevalence of *Campylobacter* in poultry flocks and carcasses is due to factors like differences in sampling collection, detection methodology, geographical location, season and production practices (Lee and Newell, 2006). *Campylobacter* counts in retail poultry meat range from 3 to 7 Log CFU/chicken (Jørgensen, et al., 2002; Manfreda, et al., 2006; Klein, et al., 2007) and *C. jejuni* is the dominant *Campylobacter* species most frequently isolated from retail poultry meat (Suzuki and Yamamoto, 2009).

Mechanism of colonization in birds

Campylobacter is naturally present in the gastrointestinal tract of poultry, more precisely in the ceca, larger intestine and cloaca (Beery, et al., 1988; Achen, et al., 1998). Most studies report that *Campylobacter* is a commensal bacterium in chickens (Beery, et al., 1988; Stern, et al., 1988; Dhillon, et al., 2006; Knudsen, et al., 2006; Smith, et al., 2008; Van Deun, et al., 2008b; Shaughnessy, et al., 2009). However, a few studies have noted a condition of vibronic hepatitis and some clinical signs like diarrhea and mortality in chicks (Lam, et al., 1992; Corry and Atabay, 2001). *Campylobacter* rapidly colonizes the ceca even with a very low dose (<50 CFU) in day of hatch chicks reaching a very high concentration in the ceca up to 10^6 - 10^8 CFU/g at the time of slaughter (Stern, et al., 1988; Stern, et al., 1995; Achen, et al., 1998; Knudsen, et al., 2006). Colonization is favored by the avian body temperature which is close to the optimum growth requirement (42°C) of thermophilic *Campylobacters* (Mead, 2002). The mechanism

behind colonization, although not fully understood, is believed to involve a dynamic process of adherence, invasion, escape from cell layers, fast replication in mucus and re-invasion which enables the bacteria to persist in the gut despite the intestinal clearance (Van Deun, et al., 2008b). Furthermore, modulation of immune response and down-regulation of expression of antimicrobial peptide genes may aid in the persistent high levels of *Campylobacter* in poultry (Meade, et al., 2009). Even though *Campylobacter* preferentially resides in the cecal crypts utilizing mucin as an energy source (Beery, et al., 1988; Hugdahl, et al., 1988), studies have reported ability to invade the liver, spleen, heart and lungs (Young, et al., 1999; Knudsen, et al., 2006; Meade, et al., 2009).

Transmission

Horizontal transmission. Poultry initially gets colonized with *Campylobacters* from environment sources such as rodents, livestock, wild birds, flies or water and birds are usually colonized by 2-3 weeks of age (Kapperud, et al., 1993; Corry and Atabay, 2001; van Gerwe, et al., 2009). Fecal shedding starts immediately after colonization leading to contamination of water, feed, and litter (Mead, 2002). Coprophagic behavior of chickens and communal source drinking water are mainly responsible for the rapid transmission throughout the flock (Keener, et al., 2004). As dry conditions of feed don't favor the survival of bacterium, feed has not been linked as a source of transmission (Newell and Fearnley, 2003). It is estimated that 2.37 new birds get colonized for every single colonized bird per day (van Gerwe, et al., 2009).

Vertical transmission. The role of vertical transmission of *Campylobacter* from poultry hen to chicks is still debatable. Various studies, however, have noted *Campylobacter* in the reproductive tracts of male and female birds, and possibly that could play a role in vertical

transmission (Pearson, et al., 1996; Buhr, et al., 2002; Cox, et al., 2002a; Cox, et al., 2002b; Donoghue, et al., 2004; Cox, et al., 2005). Experimental studies had reported that *C. jejuni* does not easily penetrate the egg shell and even if it does contaminate the egg content, would not likely survive for more than 48 h when stored at room temperature (25°C) (Doyle, 1984; Shanker, et al., 1986). One study has indicated the presence of inoculated *C. jejuni* in egg yolks up to 14 days when eggs are stored at 18°C, however, *Campylobacter* was not isolated from the eggs of broiler breeder obtained from *Campylobacter* positive commercial hatchery (Sahin, et al., 2003a). There is also a lack of evidence of *Campylobacter* in 60,000 progeny of parent breeder birds hatched from eggs of positive tested grandparent flocks (Callicott, et al., 2006). Therefore, it appears that vertical transmission either doesn't occur or occurs only rarely in chickens (Doyle, 1984; Shanker, et al., 1986; van de Giessen, et al., 1992; Jacobs-Reitsma, et al., 1995; Sahin, et al., 2003a; Callicott, et al., 2006).

PREVENTION STRATEGIES

Research on prevention of *Campylobacter* at the farm level and on the processing plant is becoming important with the rising occurrence of campylobacteriosis. Reductions in *Campylobacter* counts in pre or post-harvest poultry could significantly benefit human health as suggested by Rosenquist and co-workers (2003). They did a risk assessment and reported a potential 30 fold reduction in the human incidence of campylobacteriosis with a 2 Log reduction of *Campylobacter* counts on broiler carcasses. The European Food Safety Authority's risk assessment concluded that a 3 Log reduction in enteric *Campylobacter* counts or a 2 Log reduction on carcasses would reduce the risk of campylobacteriosis by at least 90% (EFSA Panel on Biological Hazards, 2011). These studies underscore the importance of control strategies at the farm and at the post-harvest facilities.

Pre-harvest prevention strategies

Pre-harvest prevention strategies include implementation of biosecurity measures to limit bird's exposure to the environment (Giessen, et al., 1998; Gibbens, et al., 2001; Fraser, et al., 2010; Newell, et al., 2011) and utilization of bacteriophage (Loc Carrillo, et al., 2005; Wagenaar, et al., 2005), bacteriocins (Stern, et al., 2005; Svetoch and Stern, 2010), probiotics (Morishita, et al., 1997), vaccines (Widders, et al., 1996; Buckley, et al., 2010), and feed or water supplementation of natural compounds (de Los Santos, et al., 2010; Molatová, et al., 2011) to reduce or prevent colonization in birds.

Biosecurity. Implementation of biosecurity measures such as cleaning and disinfection procedures, control of rodents, changing of footwear and washing hands before entering the farms have significant impact on reduction of *Campylobacter* prevalence in flocks (Hermans, et al., 2011b). A study conducted on Dutch broiler farms reported reduction of *Campylobacter* positive flocks from 66% to 22% on one farm and from 100% to 42% on a second farm (Giessen, et al., 1998). The application of standard methods of cleaning and disinfection, and hygiene protocols for all personnel entering the facility has shown a reduction in the prevalence in broiler flocks from 80 to <40% in the United Kingdom (Gibbens, et al., 2001). However, studies have reported that such interventions only delay the colonization and doesn't guarantee the complete elimination from flocks (Wagenaar, et al., 2006; Vandeplas, et al., 2008). *Campylobacters* are ubiquitous in the environment and hygienic measures should be implemented consistently, which is difficult to maintain (Newell, et al., 2011). Additionally, cost associated with those measures and farmer reluctance for adoption further limits the feasibility of biosecurity (Fraser, et al., 2010).

Bacteriophage. Bacteriophages are viruses, an obligate intracellular parasite lacking their own metabolism, capable of infecting and killing bacteria (Hagens and Loessner, 2007). There are two types of phage, virulent and temperate, the virulent types being able to infect and replicate in the genome, subsequently killing the host, are of particular importance for eradication of pathogens (Huff, et al., 2005). Phages are extremely host specific and *Campylobacter* specific phages have been isolated from multiple source including human, pig and poultry excreta, abattoir effluent, sewage and poultry meat (Salama, et al., 1989; Atterbury, et al., 2003b; Connerton, et al., 2004). Atterbury and colleagues (2005) reported a negative correlation between the presence of bacteriophage in ceca and *Campylobacter* counts. When chickens were dosed with phage, the amount of *Campylobacter* reduction was variable based on treatment dose, phage type, application methods, and time elapsed after administration (Loc Carrillo, et al., 2005). A significant reduction by 2 Log CFU/g in cecal *Campylobacter* counts has been reported in two studies (El-Shibiny, et al., 2009; Carvalho, et al., 2010). In a separate study, Loc Carrillo and associates (2005) determined a reduction of *Campylobacter* counts of 0.5 to 5 Log following phage treatment. Wagenaar and co-workers (2005) reported that bacteriophage treatment only delayed colonization for a week, thus proposing that poultry should only be treated a few day before slaughter (Wagenaar, et al., 2005). Research have shown that it is possible to reduce *Campylobacter* counts on chicken skin with phage application (Atterbury, et al., 2003a; Goode, et al., 2003). Although phage treatment has had no reported adverse effects on human health (Hagens and Loessner, 2010), the potential development of phage resistant *Campylobacter*, as seen in some of the studies (El-Shibiny, et al., 2009; Carvalho, et al., 2010) and consumer acceptance of poultry coated with viruses may limit its use in poultry (Janež and Loc-Carrillo, 2013).

Bacteriocins. The antimicrobial proteins or peptides produced by bacteria that either kill or inhibit the growth of other bacteria are termed bacteriocins (Cleveland, et al., 2001).

Bacteriocins are often considered natural and safe as there are no known toxic effects and act by creating pores in the target cell membrane (Cleveland, 2001; Joerger, 2003). These are produced by both gram positive and gram negative bacteria commonly belonging to the genera of *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Escherichia*, *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Pseudomonas*, and *Clostridium* (Svetoch and Stern, 2010).

Bacteriocins, B602, OR7, E760, and E50-52 produced respectively by *Paenibacillus polymyxa*, *Lactobacillus salivarius*, *Enterococcus durans/faecium/hirae* and *E. faecium* have been widely studied against *Campylobacter* in poultry (Stern, et al., 2005; Stern, et al., 2006; Line, et al., 2008; Svetoch, et al., 2008). All of these bacteriocins reduce *Campylobacter* with very high reduction in broiler and turkey poult (Stern, et al., 2005; Cole, et al., 2006; Stern, et al., 2006; Line, et al., 2008; Svetoch, et al., 2008). Studied on supplementation of E706 in drinking water of experimentally and naturally infected broilers resulted in complete elimination of bacteria in 90% of the cases; the most effective treatment reported with bacteriocin (Svetoch and Stern, 2010). However, bacteriocins require microencapsulation to protect them from proteolytic activity of host proteases, which increases the cost of these compounds (Joerger, 2003; Svetoch, et al., 2005). Although a low level of resistance of *Campylobacter* to bacteriocin has been reported both in laboratory and *in vivo* studies using a single strain, natural transformation of acquired resistance genes among the *Campylobacter* strains could potentially reduce the efficacy of these substances (Hoang, et al., 2011a; Hoang, et al., 2011b). Moreover, use of bacteriocins requires regulatory approval by the U.S. Food and Drug Administration (FDA) and presently nisin is the only approved bacteriocin in the United States (Joerger, 2003). Unfortunately, it has

been reported that nisin is ineffective against *C. jejuni* in chicken meat juice (Piskernik, et al., 2011).

Probiotics and competitive exclusion. Probiotics are live microorganisms, which confer beneficial effects on the host by enhancing the gut microflora (Fuller, 1989). The exact mechanism by which probiotics work in the gut is still not known. However, it is proposed that probiotics have several actions including production of bacteriocins, competition for nutrients, stimulation of systemic immune response, and adhering and occupying space which enables the beneficial organisms to competitively eliminate pathogens from the gut (Fooks and Gibson, 2002). Microorganisms that have been used as probiotics belong to certain bacterial species, *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *E. coli*, *Bacillus* and a variety of yeast species (Patterson and Burkholder, 2003). Prebiotics, the non-digestible substances that selectively supports the growth of beneficial bacteria, are used in combination with probiotics called synbiotics to enhance their efficacy (Gibson and Roberfroid, 1995; Patterson and Burkholder, 2003). One application of probiotics is that they are used as competitive exclusion (CE) cultures to eliminate or reduce pathogens from the poultry gut; a principal developed based on Nurmi and Ratala's research on *Salmonella* (Nurmi and Rantala, 1973). Competitive exclusion cultures have the ability to reduce *Salmonella* counts in poultry, however, inconsistent results have been reported against *Campylobacter* (Newell and Wagenaar, 2000; Humphrey, et al., 2007). Research on combination treatments in our laboratory also demonstrated the potential of some probiotic isolates to reduce enteric *Campylobacters* with mannanoligosaccharide supplementation but not with fructoligosaccharide in poultry (Arsi, 2014). The difficulty in development of CE cultures against *Campylobacter* is possibly due to the unique ecological niche that it occupies in the ceca of poultry (Newell and Wagenaar, 2000).

Vaccination. Vaccination has been a widely used method for the prevention of major poultry diseases on a commercial scale. Despite the extensive research on vaccine development against *Campylobacter*, an effective vaccine has yet to be developed commercially (Jagusztyn-Krynicka, et al., 2009). Evidence suggests that early protection of young chicks from colonization of *Campylobacter* is linked with the *Campylobacter* specific maternal antibodies (Sahin, et al., 2003b). Several researchers have reported an increase in serum IgG or mucosal IgA or both following immunization with different types of vaccine candidates leading to protection to some extent (Widders, et al., 1996; Rice, et al., 1997; Wyszynska, et al., 2004; Buckley, et al., 2010; Huang, et al., 2010; Annamalai, et al., 2013). Studies mainly focus on two types of vaccines, killed whole-cell and subunits vaccine, the latter comprising immunodominant *Campylobacter* proteins including, but not limited to, flagellin, OMP (outer membrane protein), CjaA, CjaC, and CjaD based vaccines (de Zoete, et al., 2007). The more promising results were determined with CjaA and CjaD based subunit vaccine as they elicit better humoral response with higher serum IgG and secretory IgA (Wyszynska, et al., 2004; Layton, et al., 2011). In one study, oral immunization (at day 1 and in the 2nd week) with avirulent *Salmonella* vaccine carrying *C. jejuni cjaA* gene reduced colonization by at least 6 Log CFU/g in birds challenged at 4 weeks age (Wyszynska, et al., 2004). In another study, administration of live *Salmonella* vectors expressing proteins of Cj0113 (omp18/CjaD) eliminated detectable *Campylobacter* on day 32 when birds were orally immunized on day of hatch and challenged with *Campylobacter* on day 21 (Layton, et al., 2011).

Medium chain fatty acids. Medium chain fatty acids have demonstrated antimicrobial action against different microorganisms (Bergsson, et al., 1998; Van Immerseel, et al., 2004). Caprylic acid is one of the widely studied medium chain fatty acids and is classified as generally

recognized as safe (GRAS) category by U.S. FDA (Solis de los Santos, et al., 2008b). Previous research conducted in our laboratory showed that prophylactic and therapeutic supplementation of caprylic acid in feed significantly reduced *Campylobacter* by 3-4 Log CFU/g in both 10-day of age and market age broiler chickens (Solis de los Santos, et al., 2008a; Solis de los Santos, et al., 2008b; Solis de los Santos, et al., 2009; Solís de Los Santos, et al., 2010). In a separate study, feeding of birds with encapsulated fatty acids (caprylic and capric acid in a ratio of 1:1) throughout the rearing period (42 days) consistently reduced the fecal *C. jejuni* counts (Molatová, et al., 2011). However, water supplementation of sodium salt of caprylic acids has produced inconsistent results against *Campylobacter* in chicken (Metcalf, et al., 2011). Despite the marked antimicrobial action in laboratory studies, Hermans and coworkers (2010) reported inefficacy of medium chain fatty acids when used as feed additives in birds. Hermans and coworkers (2010) suggested formulation of caprylic acid in the diet, differences in *Campylobacter* strains and bird's genetics as factors responsible for contradictory results. Similar negative results were demonstrated *in vivo* study with butyrate; one of the most bactericidal short chain fatty acids against *Campylobacter* in laboratory tests (Van Deun, et al., 2008a). The marked discrepancy in results between *in vivo* and *in vitro* studies may be due to the protective effect of mucus in the cecal crypts where *Campylobacter* resides (Van Deun, et al., 2008a; Hermans, et al., 2010).

Plant extracts. The use of plant products is increasing due to the restrictions on use of antibiotics in livestock and poultry production and shifting consumer preferences towards naturally occurring compounds (Burt, 2004; European Commission, 2005). Several compounds from plants including, but not limited to, phenols, quinones, flavones, tannins, coumarins, terpenoids, essential oils, alkaloids, and lectins possess antimicrobial properties against different

microorganism (Cowan, 1999). Various studies have been conducted using plant compounds to control *Campylobacter* in both pre-harvest and post-harvest poultry. *In vitro* studies have demonstrated antibacterial action of essential oils, such as trans-cinnamaldehyde, eugenol, carvacrol, and thymol against *C. jejuni* in cecal contents (Johny, et al., 2010a). However, these compounds fail to demonstrate a consistent reduction in cecal *Campylobacter* counts in chicken studies (Metcalf, 2008; Hermans, et al., 2011a; Arsi, et al., 2014). Earlier research in our laboratory showed at least a 5 Log reduction in *Campylobacter* counts with 2% or 4% cranberry extract *in vitro*, however, neither dose was able to reduce *Campylobacter* in the bird trial (Woo-Ming, 2012). One experiment has determined antimicrobial activity of 9 out of 28 different fruit extracts with plum, lime and orange peel showing the greatest efficacy against *Campylobacter* (Valtierra-Rodríguez, et al., 2010). When mixtures of plum, lime and orange peel were applied to chicken skin inoculated with *C. jejuni* (10^5 CFU/mL) and incubated for 48 h at 4°C, it completely eliminated detectable *Campylobacter* (Valtierra-Rodríguez, et al., 2010). The combination of rosemary extract and pre-freezing (-20°C for 24 h) has shown synergistic effect reducing *Campylobacter* counts by greater than 2 or 3 Log in chicken meat or juice, respectively (Piskernik, et al., 2011). Another study has reported the action of several other essential oils including marigold, ginger root, jasmine, carrot seed, celery seed, mugwort, patchouli, gardenia, spikenard, and cedarwood against *C. jejuni* (Friedman, et al., 2002). Research has also determined the antibacterial response of different types of tea, lemon, turmeric, and Chinese leek against *Campylobacter* (Lee, et al., 2004; Murali, et al., 2012). Further in depth research is still warranted to figure out the right dose and application method in commercial conditions.

Post-harvest prevention strategies

Campylobacter contamination is a serious problem in processing plants and this contamination differs in various steps in processing including scalding, de-feathering, evisceration, and chilling. A study determined the prevalence of *Campylobacter* contamination in the plant of 53.3%, 66.7%, 40% and 33.3% in carcasses after scalding and defeathering, evisceration, cooling and in finished breast meat with skin respectively (Klein, et al., 2007). Because of this occurrence of contamination, appropriate post-harvest strategies are necessary to control *Campylobacter* prevalence in retail poultry products. Post-harvest interventions are divided broadly into physical and chemical decontamination methods. Physical methods are comprised of steam, water, electrolyzed water, pressurized water, ozonated water, irradiation, ultrasound, air chilling and freezing (Loretz, et al., 2010). It has been reported that steam, hot water and electrolyzed water reduced *Campylobacter* 2.3-3.8, 0.9-2.1 or 1.1-2.3 Log, respectively in processed poultry (Loretz, et al., 2010). But these treatments can adversely effect carcass organoleptic properties (Dawson, et al., 1963; Cox, et al., 1974; Notermans and Kampelmacher, 1974; McMeekin and Thomas, 1978; Thomas and McMeekin, 1982; Whyte, et al., 2003). Boysen and Rosenquist (2009) revealed the effectiveness of freezing over air chilling, crust-freezing and steam-ultrasound. Even though a reduction in counts was obtained with freezing (-20°C), *Campylobacters* were recovered from the frozen sample and still present a risk for campylobacteriosis (Bhaduri and Cottrell, 2004). It was estimated that short term freezing (-30°C) of chicken wings for 72 h reduces *Campylobacter* by 1.8 Log CFU/g, however, the condition used in poultry processing to superchill carcass to nonfrozen-state are not likely to reduce the counts significantly (Zhao, et al., 2003).

Chemical decontamination includes organic acids and chlorine based treatments. Reidel and coworkers (2009) have evaluated efficacy of 11 chemicals to reduce *Campylobacter* on chicken skin. When chicken skin was dipped for 1 min, they found the most effective treatment with cetylpyridinium chloride (0.5%) and benzalkonium chloride (1%) resulting in at least 4.2 Log CFU/mL reduction in counts. Formic acid (2%), lactic acid (2.5%), trisodium phosphate (10%), capric acid sodium salt (5%), and grapefruit seed extract (1.6%) also reduced *Campylobacter* in a range from 1.75 to 3.8 Log CFU/mL (Riedel, et al., 2009). Comparable reductions (2-2.7 Log CFU/mL) were determined when chicken legs were dipped for 1 min in 20mM monocaprin (0.5%) (Thormar, et al., 2011). In a separate study, use of a combination of peracetic acid and hydrogen peroxide (85ppm) in poultry chillers decreases the *Campylobacter* positive carcasses by 43 % (Bauermeister, et al., 2008). However, studies have reported discoloration of skin when breast skin is dipped in different organic acids like acetic acid, citric acid, lactic acid, malic acid, mandelic acid and tartaric acid (Bilgili, et al., 1998). Residues in meat, problem in disposing of chemicals and their high costs are other problems associated with chemical treatments (SCVPH, 1998; EFSA BIOHAZ Panel, 2014). It should be taken into account that chemicals should be approved, have well documented efficacy, level and contact time suitable to use in processing plants, be cost-effective and should not produce any harmful effects on product quality to be applicable in industry (Bauermeister, et al., 2008).

USE OF β -RESORCYLIC ACID IN PRE-HARVEST AND POST-HARVEST POULTRY

β -resorcylic acid (BR) is a phenolic compound and as a secondary metabolite plays a crucial role in biochemistry and physiology of several plant species (Friedman, et al., 2003). This compound is used in foods as a flavoring substance (European Food Safety Authority, Ref no. 00910) and classified under the category of “Everything Added to Food in the United States”

by the US Food and Drug Administration (EAF 3045, CAS RN 89-86-1). Commercially, it is available in powder form as 2-4, dihydroxybenzoic acid (CAS: 89-86-1, Sigma-Aldrich Co., MO, USA). Beta-resorcylic acid is soluble in hot water, alcohol, ether and olive oil and boiling often results in loss of CO₂ (O'Neil, et al., 2001).

Mechanism of action

The exact mechanism by which BR kills or inhibits the growth of microorganisms is not well known and knowledge regarding antimicrobial action is limited by lack of specific research related to this compound. However, being a weak acid, like benzoic acids, it is thought to act in an undissociated form which diffuses passively through bacterial cell membranes and causes a pH imbalance leading to disruption of biochemical processes and uncoupling of cellular energy production (Friedman, et al., 2003; Baskaran, et al., 2013b).

Research on β -resorcylic acid

Early research in the 1950s evaluated BR for its antimalarial activity against *Plasmodium lophurae* in Leghorn chicks (Thompson, et al., 1953). When this compound was administered with salicylic acid in rats infected with *Trypanosoma lewisi*, it reversed the activity of salicylic acid resulting in reduction of *Trypanosoma* population in blood but did not exhibit any antiablastic activity itself (Becker, 1961). Further study has demonstrated the antibacterial activity of BR against *C. jejuni*, *in vitro* (Friedman, et al., 2003). In a recent study, it was determined that BR increased the sensitivity of *Salmonella* Typhumurium H3380 to ampicillin, chloramphenicol, streptomycin, and sulfonamide (Johnny, et al., 2010b). Mattson and colleagues (2011) reported concentration-dependent action of BR when tomatoes inoculated with *Salmonella* (10⁸CFU) were dipped for 1 min in BR treatment solution. *Salmonella* was reduced

to less than 1.0 Log CFU/mL and 3.0 Log CFU/mL respectively by 1% and 0.75% BR after 1 min exposure, however, both doses decreased this pathogen to undetectable levels at 3 min exposure without change in color (Mattson, et al., 2011). In another study, the same dose (1%) of BR, even at 1 min exposure, eliminated detectable counts of *E. coli* 0157:H7 when used for washing of infected apples (6.6 Log CFU/apple; Baskaran, et al., 2013b). Research on BR against *E. coli* 0157:H7 in decontamination of cattle hides has been shown to be more effectiveness by spraying of 1% BR solution on hides at 60°C than at 23°C (Baskaran, et al., 2013a). This compound was also tested against *Listeria monocytogenes* in frankfurters. Dipping of frankfurter experimentally infected with *L. monocytogenes* (6 Log CFU/frankfurter) in 1.5% BR solution at 65°C for 30 s consistently reduced this bacterium during storage and eliminated detectable counts by the end of storage period on day 70 (Upadhyay, et al., 2013).

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CHAPTER 2: Efficacy of β -resorcylic Acid to Reduce *Campylobacter jejuni* in Pre-harvest and Post-harvest Poultry

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ABSTRACT

Campylobacteriosis is one of the leading foodborne illnesses in United States, and is associated with the consumption of poultry and poultry products. Reducing *Campylobacter* in these species will reduce the burden of this disease. Unfortunately, most strategies employed to reduce *Campylobacter* in chicken have either not been successful or produced inconsistent results. One potential control strategy is the use of β -resorcylic acid (BR), a phytophenolic compound classified by the US FDA as “Everything Added to Food in the United States” (EAF 3045) and is therefore deemed safe for consumption. This compounds has antibacterial activity against *Salmonella*, however, its efficacy to control *Campylobacter* in poultry has not been evaluated. Preliminary studies in our laboratory demonstrated that BR kills *Campylobacter jejuni*, *in vitro*. Therefore, the objective of this study was to evaluate whether BR would reduce *Campylobacter* in chickens. In pre-harvest studies, day of hatch chicks were fed one of five treatments (0, 0.25, 0.5, 1 or 2%BR) in the first trial, whereas a second trial was conducted including two additional doses of 0.75% & 1.5% BR (n=10 chicks/dose). Birds were challenged with mixture of four wild strains of *C. jejuni* ($\sim 10^6$ CFU/mL) on day 7 and cecal samples were collected on day 14 and enumerated for *Campylobacter*. In post-harvest studies four trials, two each on thigh skin and breast meat, were conducted. Chicken skin or meat samples (2 ± 0.1 g) were inoculated with 50 μ L of *C. jejuni* ($\sim 10^7$ CFU/mL). Following 30 min of attachment, samples were dipped into their respective treatment solutions (0, 0.5, 1, 2% BR) for 30 s and suspended 2 min (n=10 samples/dose) and evaluated for reduction in *Campylobacter* counts. Data were analyzed by PROC GLM procedure of SAS ($P < 0.05$). *Campylobacter* counts were reduced by 1.4 Log CFU/g for the 2% dose in the first pre-harvest trial and by 4.2 or 2.8 Log CFU/g for the 0.5 % & 1% BR doses in the second pre-harvest trial. In the post-harvest studies,

all doses of BR significantly reduced *Campylobacter* counts in both meat and skin. Results of these experiments suggest post-harvest application of BR is the most effective treatment and may help reduce the incidence of human campylobacteriosis.

Key words: *Campylobacter jejuni*, β -resorcylic acid, pre-harvest, post-harvest, chicken.

INTRODUCTION

Campylobacter contamination of food products is the leading causes of foodborne illness worldwide (Altekruse, et al., 1999; Hall, et al., 2005; WHO, 2012; CDC, 2014; EFSA, 2013). *Campylobacter*, in particular *Campylobacter jejuni*, is the second most commonly reported foodborne pathogen in the United States (*Salmonella* is the first) in causing an estimated 850,000 illnesses and 13,256 quality-adjusted life year loss per year (Scallan, et al., 2011; Batz, et al., 2014; CDC, 2014). Actual cases are probably higher than these estimates due to under-reporting or sick individuals not seeking medical attention (Skirrow, 1991; Mead, et al., 1999; Samuel, et al., 2004). *Campylobacter* has been reported to cause mild to severe gastro-enteritis and occasionally results in Guillain-Barré syndrome, reactive arthritis, or irritable bowel syndrome (Berden, et al., 1979; Rhodes and Tattersfield, 1982; Spiller, 2007; Gradel, et al., 2009). Studies have indicated that improper handling or consumption of chicken or other food products cross contaminated with poultry meat or juices during food preparation as a common cause of campylobacteriosis (Altekruse, et al., 1999; Rosenquist, et al., 2003; Friedman, et al., 2004a; Danis, et al., 2009; Lindmark, et al., 2009; Lubert, 2009; Fajó-Pascual, et al., 2010).

Campylobacter spp is normally colonized in the gastrointestinal tract of chickens by the third to fourth week of age and is a commensal organism in poultry (Annan-Prah and Janc, 1988; Stern, et al., 1988; Humphery, et al., 1993; Dhillon, et al., 2006;). Various studies have reported enteric colonization up to 10^6 - 10^8 colony forming unit (CFU)/g of cecal material (Achen, et al., 1998; Beery, et al., 1988) and concluded that high fecal contamination can occur during transport and during slaughter (Berrang, et al., 2000; Herman, et al., 2003; Reich, et al., 2008). Up to 90% of retail chicken carcasses have detectable *Campylobacter* contamination (Stern, et al., 2001; Wong, et al., 2007) ranging from 3 to 7 Log CFU/per chicken (Jørgensen, et al., 2002;

Manfreda, et al., 2006; Klein, et al., 2007). As *C. jejuni* has a low infective dose of approximately 400 cells (Black, et al., 1988), poultry products present a high risk of *Campylobacter* infections in humans. Therefore, studies have emphasized the importance of controlling *Campylobacter* in poultry flocks at the farm level and on carcasses during processing in order to reduce the incidence of campylobacteriosis (Rosenquist, et al., 2003; Arsenault, et al., 2007; Reich, et al., 2008).

Current approaches for reduction of *Campylobacter* in poultry and poultry products include both pre- and post-harvest interventions. Different strategies have been tried to eliminate or reduce *Campylobacter* in pre-harvest poultry including; implementation of strict biosecurity to limit bird's exposure to environmental sources (Giessen, et al., 1998; Gibbens, et al., 2001; Fraser, et al., 2010; Newell, et al., 2011) treating with bacteriophages (Loc Carrillo, et al., 2005; Wagenaar, et al., 2005) bacteriocins (Stern, et al., 2005; Svetoch and Stern, 2010) prebiotics (Morishita, et al., 1997; Arsi, 2014) vaccines (Widders, et al., 1996; Buckley, et al., 2010) or natural compounds (Solis de los Santos, et al., 2010; Molatová, et al., 2011; Woo-Ming, 2012; Arsi, et al., 2014). Unfortunately, most of these treatments have had limited success or produced inconsistent results.

Researchers have also tried to reduce *Campylobacter* on broiler carcasses by physical and chemical methods in post-harvest poultry. Chemical interventions studied have included use of organic acids, chlorine or phosphate based treatments (Zhao and Doyle, 2006; Bauermeister, et al., 2008; Riedel, et al., 2009; Birk, et al., 2010; Thormar, et al., 2011). Although these interventions yielded reductions in the range from 1 to 2.2 Log (Loretz, et al., 2010), acceptance has been limited due to residues or discoloration of meat, consumer preference for meat without additives, disposing of the hazardous waste and the high cost of treatment (Bilgili, et al., 1998;

SCVPH, 1998; EFSA BIOHAZ Panel, 2014). Physical methods like freezing, hot water or steam, electrolyzed water, irradiation, ultrasound, and air chilling have also been tested (Patterson, 1995; Park, et al., 2002; Corry, et al., 2007; James, et al., 2007; Musavian, et al., 2014). Various studies have reported that hot water (>60°C) or steam exert adverse effects on carcass appearance (Dawson, et al., 1963; Cox, et al., 1974; Whyte, et al., 2003). Immersion can cause swelling of skin and tissue allowing bacteria to move deeper into the tissue making their removal more difficult (Notermans and Kampelmacher, 1974; McMeekin and Thomas, 1978; Thomas and McMeekin, 1982) and *Campylobacter* survived even after freezing (Bhaduri and Cottrell, 2004). Furthermore, Boysen and colleagues (2013) have reported that reductions observed after inoculating carcasses with just one *Campylobacter* strain are not representative of carcasses naturally exposed to multiple *Campylobacter* species during production or in the processing plant. Therefore, our post-harvest part of this studies was designed using four strains of *C. jejuni* and chicken skin and meat samples were inoculated to obtain a more representative contamination scenario.

The use of plant antimicrobials has gained renewed attention with the restriction of antibiotics in livestock production worldwide (European Commission, 2005; Perkins, 2012). In this study, we evaluated the ability of the natural plant compound, β -resorcylic acid (CAS: 89-86-1, Sigma-Aldrich Co., MO, USA) to reduce *Campylobacter* in both pre- and post-harvest poultry. Beta-resorcylic acid (BR; 2, 4 dihydroxybenzoic acid) is a phytophenolic compound widely distributed among the angiosperms and is a secondary metabolite that plays a key role in the biochemistry and physiology of plants (Friedman, et al., 2003). This compound is used in food as a flavoring substance (European Food Safety Authority, Ref no. 00910) and classified under the category of “Everything Added to Food in the United States” by the US Food and

Drug Administration (EAF 3045, CAS RN 89-86-1). *In vitro* antibacterial activity of BR has been reported for *C. jejuni* (Friedman, et al., 2003), *Salmonella spp* (Mattson, et al., 2011), *E.coli* (Friedman, et al., 2004b; Baskaran, et al., 2013a) and *Staphylococcus aureus* (Friedman, et al., 2004b; Alves, et al., 2013). However, this is the first study to our knowledge in which BR has been evaluated for its ability to reduce *Campylobacter* both in pre-harvest and post-harvest poultry.

MATERIALS AND METHODS

In vitro efficacy of β -resorcylic acid on *Campylobacter*

An *in vitro* experiment was conducted to confirm the antibacterial activity of BR against *C. jejuni* in the presence of cecal material. *C. jejuni* was prepared as per the methods previously described in our laboratory (Woo-Ming, 2012; Arsi, et al., 2014). Briefly, 10 μ L glycerol stocks of four wild strains of *C. jejuni* were cultured into 5mL *Campylobacter* enrichment broth (CEB, International Diagnostics Group, Bury, Lancashire, UK) and incubated at 42°C under microaerophilic condition (85% N₂, 10% CO₂, 5% O₂) for 48 h. Subcultures of each strain were made in 5mL CEB and again incubated at 42°C under microaerophilic condition for 24 h. A wet mount preparation was made to check motility and viability under the microscope and all strains were mixed in a tube and centrifuge at 3000rpm for 10 min at 21°C. Supernatant solutions was discarded and cells were resuspended with equal volume of Butterfield's phosphate diluent (BPD). The *C. jejuni* suspension was inoculated into pooled and autoclaved cecal content in a tube to obtain $\sim 2 \times 10^7$ CFU/mL. Different treatment solutions were prepared by dissolving appropriated quantities of BR in CEB to obtain final concentration of 20, 30, 40, 50, 60, 70, and 75mM. CEB was used as control solution. Then, 100 μ L of cecal content inoculated with *C.*

jejuni and 900 μ L of respective treatment solutions were added in separated tubes and incubated at 42°C under microaerophilic condition for 0, 8, and 24 h. Triplicates of each samples were made and serially diluted (1:10) in BPD. Samples were then plated in Campy line agar (CLA; Line, 2001) and incubated at 42°C under microaerophilic condition. *Campylobacter* colonies were enumerated after 48 h.

Pre-harvest efficacy of β -resorcylic acid supplemented in chicken feed

Experimental Design. Day of hatch broiler chicks (Cobb500) were obtained from a local commercial hatchery. Birds were weighed individually, randomly allocated into different groups and placed on separate floor-pens with pine shavings. Two trials were conducted with five treatments groups (0, 0.25%, 0.5%, 1%, 2% BR) in the first trial (n=10 chicks/treatment) and two additional treatments (0.75%, 1.5% BR), based on result of trial 1 in an attempt to determine the optimal dose for this compound, with a total of seven treatments in the second trial (n=10 chicks/treatment). Beta-resorcylic acid was mixed in feed and birds were fed ad libitum water and treated or untreated feeds throughout the 14 day study period. Feed consumption, initial and final body weights of individual birds were recorded during this study.

Campylobacter challenge and enumeration. Birds were challenged via oral gavage with four wild strains of *Campylobacter jejuni* on day 7. The *Campylobacter* challenge in BPD solution was prepared by a method previously used in our laboratory and described above for our *in vitro* study (Woo-Ming, 2012; Arsi, et al., 2014). To quantitate the dose given to each chick, the inoculum was serially diluted (1:10) and plated on CLA (Line, 2001) plates. The cultured plates were incubated at 42°C for 48 h under microaerophilic conditions for quantification of

challenge dose. The challenge dose was approximately 10^6 CFU/mL for both trials and each bird was inoculated with 0.25mL of the challenge solution.

A method previously described in our laboratory was used for cecal *Campylobacter* enumeration (Cole, et al., 2006). On day 14, ceca were collected aseptically in sterile sample bags and the cecal contents were serially diluted and each dilution was subsequently plated on CLA for enumeration of *Campylobacter*. Presumptive *Campylobacter* colonies were directly enumerated and confirmation were made with a latex agglutination test (SCIMEDX Co., NJ, USA).

Post-harvest efficacy of β -resorcylic acid on chicken skin and meat samples

Sample Preparation. For the post-harvest trials, chicken was purchased from local grocery store and 2 g samples of skin (from chicken thigh) and meat (from breast meat) were prepared aseptically and stored at -20°C. During each trial, the samples were thawed and randomly allocated to each treatment group.

Experimental design. A total of four trials, two each on skin or meat, were conducted. This experiment consisted of four treatment groups (0, 0.5, 1, & 2% BR), each trial having ten skin or meat samples. The treatment solution was made in BPD at 42°C for better solubility (Budavari, 1989). First, samples were thawed to room temperature and a 50 μ L ($\sim 10^7$ CFU/mL) of a cocktail of four wild strains of *Campylobacter jejuni* were inoculated onto meat or skin samples. After inoculation, the samples were kept at room temperature for 30 min to adhere bacteria onto the samples. All samples for each treatment group were dipped simultaneously into 500mL of respective BR treatment or control solution for 30 s and dripped dried for 2 min. Each individual sample was then immediately transferred to separate tube with 18 mL of BPD,

vortexed, and the suspension was serially diluted in BPD, plated on Campy line agar (Line, 2001), and incubated at 42⁰ C for 48 h under microaerophilic conditions. Direct colony counts were recorded and converted into CFU/g of sample.

STATISTICAL ANALYSIS

The *Campylobacter* mean CFUs were logarithmic transformed (Log CFU/g) to maintain the homogeneity of variance (Byrd, et al., 2001). The data were analyzed by using PROC GLM procedure of SAS 9.3 (SAS Institute, 2010). The treatment means were partitioned by LSMEANS analysis and a probability of P<0.05 required for statistical significance.

RESULTS

In vitro efficacy of β -resorcylic acid on Campylobacter

There were significant reductions (4.7, 3.6, 5.1, 6.1 Log CFU/g) of *Campylobacter* counts *in vitro* study with cecal material at 0 h by 50, 60, 70, and 75mM concentrations of BR respectively. All the concentrations (20, 30, 40, 50, 60, 70, 75mM) of BR showed significant reductions by at least 4.6 Log CFU/g after 8 and 24 h compared to control (Figure 1).

Pre-harvest efficacy of β -resorcylic acid supplemented in chicken feed

In our pre-harvest studies, cecal *Campylobacter* counts were reduced by 1.4 Log CFU/g in birds fed with 2% dose of BR in the first trial and by 4.2 Log CFU/g or 2.8 Log CFU/g for the birds fed 0.5% or 1% BR doses in the second trial when compared to *Campylobacter* control birds (Table 1). Body weight gain was significantly reduced in the birds fed 0.5, 1 and 2% doses of BR in the first trial and by 2% BR in the second trial when compared to controls. Total feed

consumption was reduced for the 2% BR group in trial 1 and all treatment groups in trial 2 when compared with controls (Table 2).

Post-harvest efficacy of β -resorcylic acid on chicken skin and meat samples

In the post-harvest studies, *Campylobacter* counts on meat or skin were significantly reduced for all doses of BR tested compared to the control in all the trials (Figure 2 & 3). The greatest reduction on meat was for the 2% BR dose in trial 1 (3.1 Log CFU/g) and either 1% or 2% in trial 2 (1.9 Log CFU/g) when compared with controls. On the skin samples, all three concentration of BR tested produced comparable reductions (1-1.2 Log CFU/g) in trial 1 and the 2% dose had the greatest reduction (2.1 Log CFU/g) in trial 2 when compared with controls.

DISCUSSION

In vitro testing demonstrated that BR is effective at reducing or eliminating detectable *Campylobacter* counts in liquid media containing cecal contents. The higher doses of BR (50-75mM) produced an immediate reduction in counts after dosing (0 h) and reduced counts for the 20mM dose or eliminated detectable *Campylobacter* counts for all the other doses (30-75mM) at 8 or 24 h, respectively. These results are consistent with that of Friedman and coworkers (2003) and they speculated this was due to a change in pH, which alters the electrochemical balance of bacterial cell facilitating cell death.

In the first pre-harvest trial using 0.25, 0.5, 1.0 or 2.0 % BR in the feed, enteric *Campylobacter* counts were reduced for only the highest dose (2%) of BR when compared with positive *Campylobacter* controls. In the second trial, the additional doses of 0.75 and 1.5% BR were added to the study in an attempt to determine the optimal dose for this compound. In this

trial, however, only the 0.5 and 1% but not the 2% doses of BR reduced enteric *Campylobacter* counts.

It is unclear why there wasn't consistent efficacy when dosing with BR in this pre-harvest study. It is possible that there is variability of absorption of BR in individual bird's digestive tracts. This could potentially produce different BR concentrations reaching the ceca and subsequently impacting its efficacy on cecal *Campylobacter* colonization. There are few studies on the absorption, metabolism, and mechanism of BR. Beta-resorcylic acid has a moderate dissociation constant (pKa 3.30) similar to benzoic acid (pKa 4.20) and as a weak acid remain in a non-dissociated form in the stomach and intestine (Mattoo, 1959; Milne, 1965). Weak acids in non-dissociated form are easily absorbed in acidic medium (Milne, et al., 1958). These reports might support the differences in BR efficacy due to absorption in the G.I tract. Moreover, *in vitro* studies on anaerobic metabolism of resorcylic acid by fermenting bacteria such as *Clostridium* spp. in a co-culture with *Campylobacter* spp. sequentially cleaved into non-aromatic compounds (Kluge, et al., 1990). Birds have diverse gut microflora and *Clostridium* can be found in the small intestine of young chicks (Amit-Romach, et al., 2004; Apajalahti, et al., 2004). Thus, there is a chance of degrading this compound into different compounds which may or may not possess antibacterial properties. One possible solution to these potential issues is encapsulating BR to prevent quick absorption or rapid metabolism by other microflora.

It is also reported that domestic fowl excrete benzoic acid and other aromatic acids in conjugation with ornithine, a compound derived from dietary arginine (Nesheim and Garlich, 1963). A study on supplementation of 0.2% benzoic acids in broiler chickens reported arginine deficiency related reduction in growth performance (Józefiak, et al., 2010). Thus, a similar situation in which absorbed BR may lead to deficiency of arginine and impair the broiler

performance as seen in 2% BR inclusion. Upadhayaya et al (2014) had also reported the decreased in body weight gain with 1% BR when used to reduce colonization of *Salmonella* Enteritidis in 21-day broiler chickens. Reduction of body weight gain, as also observed in the current study (Table 2), rules out the possibility of increasing the BR dose to possibly improve its efficacy against *Campylobacter* due a concomitant reduction in production performance.

Another potential cause for this variability is differences in each individual bird's microbiome, not only within each trial but between trials. Although it would be possible to eliminate this variable by raising gnotobiotic birds in environmentally sterile isolators, unfortunately it is not economically feasible for poultry producers to raise birds under these conditions. Therefore, any treatment developed for commercial poultry production has to be rugged enough to reduce *Campylobacter* counts in birds acquiring microbes from diverse environments. Treatment variability is not limited to the current study. Previous reports from our laboratory (Metcalf, et. al., 2011; Woo-Ming, 2012; Arambel, 2014; Arsi, et. al., 2014) and others researchers (Hakkinen and Schneitz, 1999; Hermans, et. al., 2010; Robyn, et. al., 2013) have also reported variable results when evaluating treatment against *Campylobacter* in pre-harvest poultry.

Beta-resorcylic acid treatment rapidly reduced *Campylobacter* counts on both skin and meat samples when tested a few minutes after application (Figures 2 and 3). The greatest reduction on meat was for with 2% dose in trial 1 (3.1 Log CFU/g) and either the 1 or 2% dose in trial 2 (1.9 Log CFU/g). On the skin samples, all three doses reduced *Campylobacter* counts in trial 1 (1-1.2 Log CFU/g) but the 2% dose provide the greatest reduction in trial 2 (2.1 Log CFU/g). A reduction by BR on both skin and meat is important since both skin and meat can be highly contaminated with *Campylobacter* (Hansson, et al., 2015; Jørgensen, et al., 2002).

The reduction reported in this study are supported by other researcher evaluating post-harvest BR treatments on foods. Baskaran and co-workers (2013b) washed apples with 0.5 % (pH 1.40) and 1% (pH 1.40) BR and reported undetectable counts of *E. coli* O157:H7 after a 5 min exposure. Treatment with one percent BR was more effective against *E. coli* O157:H7 in treatment of cattle hides at 60⁰C (4 Log CFU/cm²) than at 23⁰ C (3 Log CFU/cm²) with no significant difference between the 2 min and 5 min exposure time (Baskaran, et al., 2013a). *Salmonella spp* were significantly reduced on tomatoes washed with deionized water containing 0.75 or 1% BR following a 1 min exposure time (Mattson, et al., 2011). The reduction in *Campylobacter* counts reported in the current study are similar to the reduction (1.57-1.78 Log CFU/mL) reported with formic acid (2%), lactic acid (2.5%), trisodium phosphate (10%), and capric acid sodium salt (5%) but not as great as the use of cetylpyridinium chloride (0.5%) and benzalkonium chloride (1%) (>4.2 Log CFU/mL) (Riedel, et al., 2009).

In conclusion, application of BR in pre-harvest poultry produced an inconsistent reduction in enteric *Campylobacter* colonization but post-harvest application produced a consistent reduction on both skin and meat post-harvest samples. Follow-up testing is needed to evaluate the effect of BR on spoilage bacteria and organoleptic properties. However, the reduction of BR on post-harvest poultry samples supports the potential evaluation of BR as part of a multi-faceted strategy to reduce the incidence of this important human foodborne pathogen.

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Table 1: The effect of different concentrations of BR on cecal *Campylobacter* counts (Log CFU/g of cecal content) in 14 days old broiler chickens (Mean \pm SEM)¹.

Treatments	Trial 1	Trial 2
<i>Campylobacter</i> Control	7.47 \pm 0.28 ^{ab}	7.27 \pm 0.42 ^{ab}
<u>β-resorcylic acid</u>		
0.25%	8.04 \pm 0.21 ^a	5.50 \pm 1.22 ^{abc}
0.5%	6.82 \pm 0.46 ^{bc}	3.03 \pm 0.88 ^d
0.75%	-	7.61 \pm 0.44 ^a
1%	6.90 \pm 0.18 ^{bc}	4.47 \pm 0.92 ^{cd}
1.5%	-	5.22 \pm 0.81 ^{bcd}
2%	6.09 \pm 0.48 ^c	7.74 \pm 0.46 ^a

^{a, b, c} Means within columns with no common superscripts differ significantly (P<0.05).

¹Chicks were fed with BR in all groups except *Campylobacter* controls (0% BR) from day old hatch to the end of 14-day studies in both the trials. On day 7, birds were challenged via orally gavage (0.25mL/birds) with approximately 1×10^6 CFU/mL of mixture of four wild strains of *Campylobacter jejuni*. *Campylobacter* data were Log₁₀ transformed for statistical analysis.

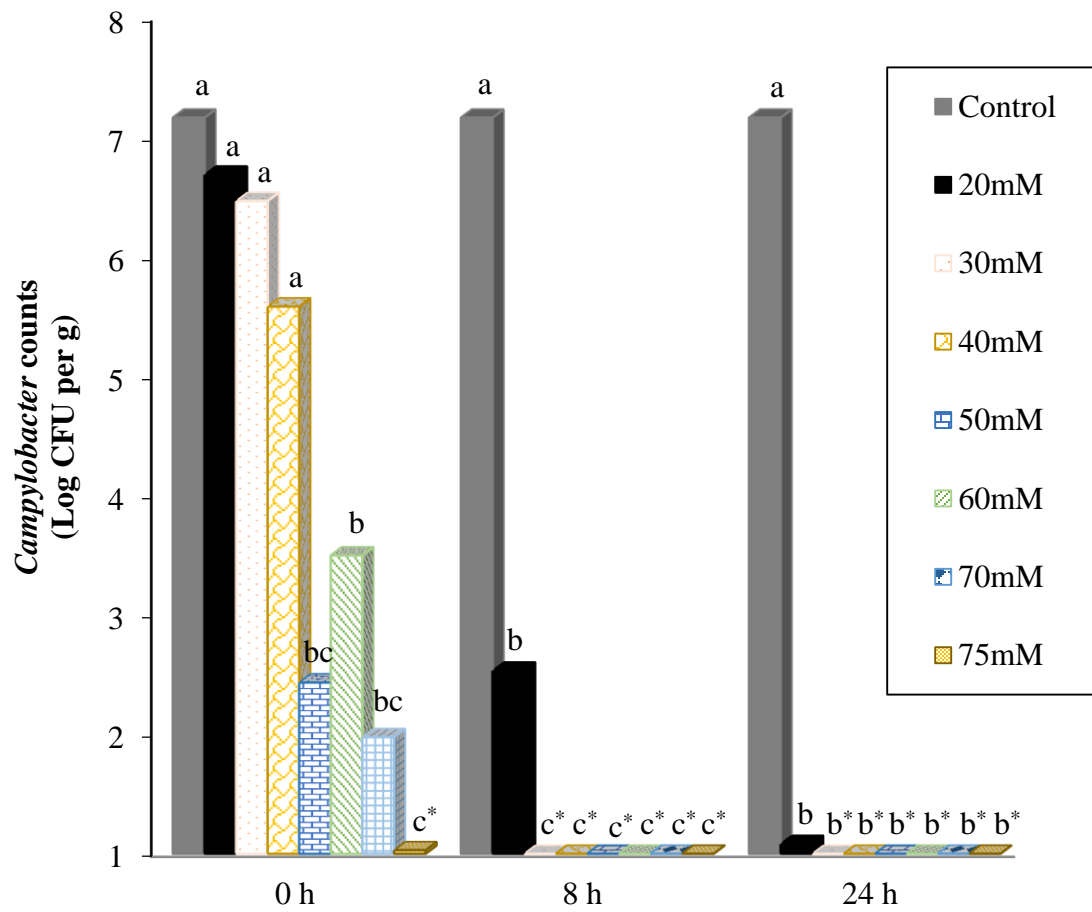
Table 2: The effect of different concentrations of BR on body weight gain (Mean \pm SEM) and feed consumption (g/bird) in 14 days old broiler chickens¹.

	Trial 1		Trial 2	
	Body weight gain (g)	Feed consumption (g)	Body weight gain (g)	Feed consumption (g)
Positive Control	325.68 \pm 14.08 ^a	502.3	263.06 \pm 27.58 ^{ab}	735.9
<u>β-resorcylic acid</u>				
0.25%	279.67 \pm 27.17 ^{ab}	622.2	269.33 \pm 21.45 ^{ab}	604.4
0.5%	253.74 \pm 23.34 ^{bc}	518.4	283.87 \pm 24.51 ^a	546.2
0.75%	-	-	288.53 \pm 20.10 ^a	620.6
1%	228.40 \pm 21.93 ^{bc}	624.8	310.53 \pm 20.37 ^a	650
1.5%	-	-	207.6 \pm 24.51 ^{bc}	650.7
2%	212.63 \pm 4.43 ^c	438.8	150.99 \pm 24.82 ^c	575.2

^{a, b, c} Means within columns with no common superscripts differ significantly (P<0.05).

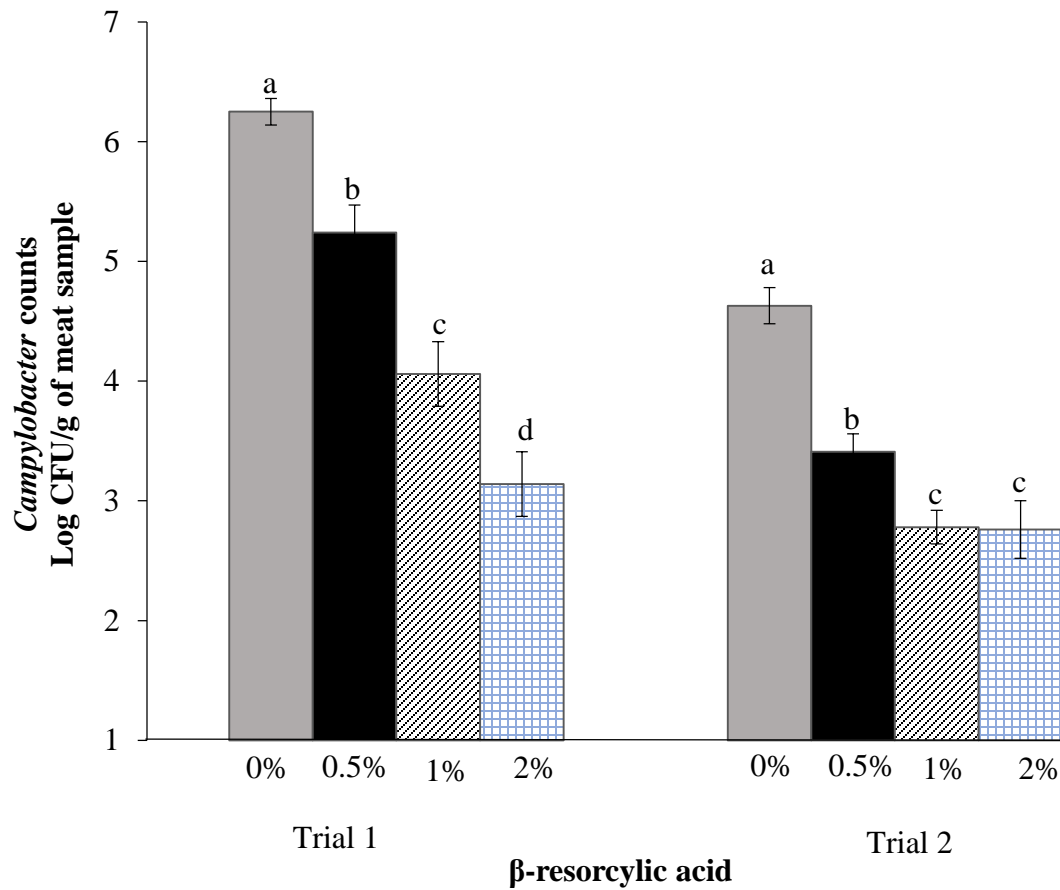
¹Chicks were fed with BR in feed in all groups except control from day old hatch to the end of 14-day studies in both the trials.

Figure 1: The effect of different concentrations (20, 30, 40, 50, 60, 70, and 75mM) of BR on *in vitro* growth of *Campylobacter jejuni* in cecal material (Mean \pm SEM).



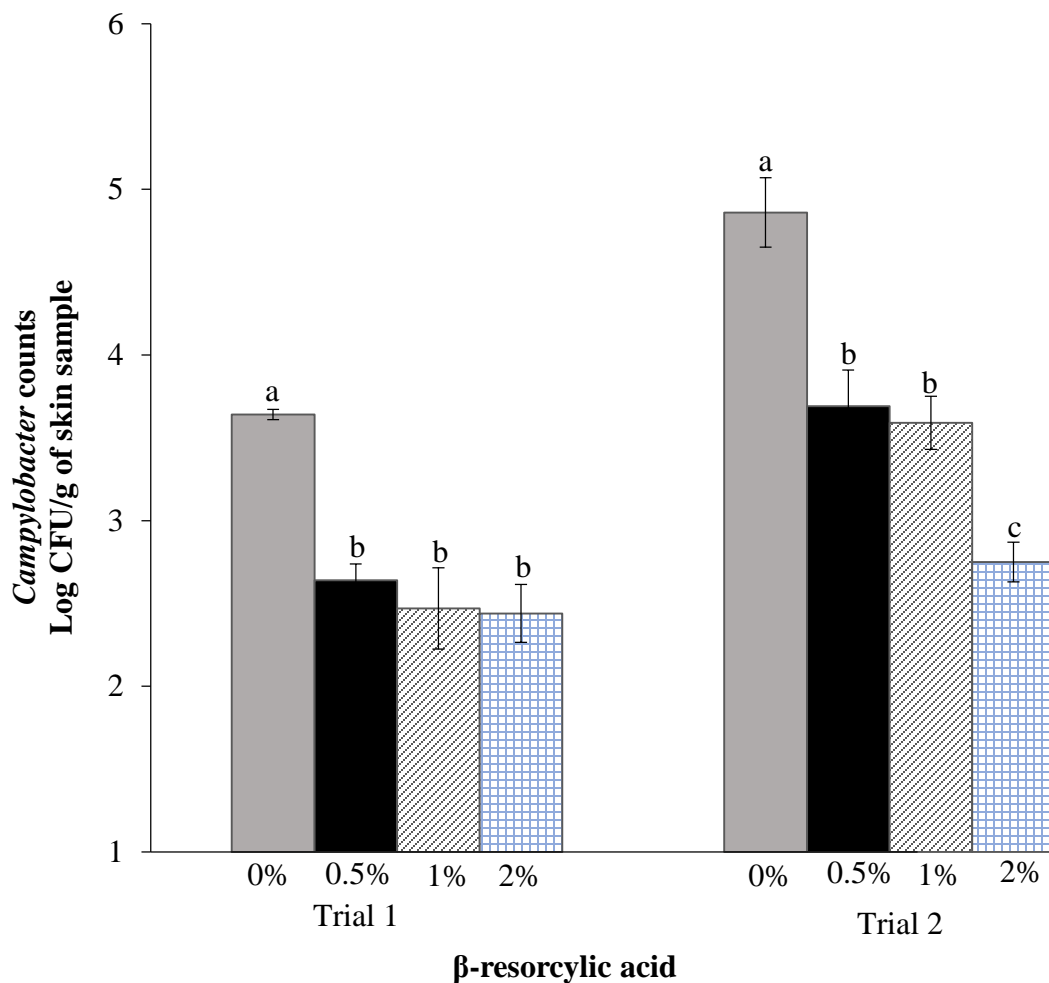
^{a, b, c} Means within hours with no common superscripts differ significantly ($P < 0.05$). All *Campylobacter* data were Log_{10} transformed for statistical analysis.

Figure 2: The effect of different concentrations of BR on chicken meat samples ($2\pm 0.1\text{g}$) challenged with approximately 1.7×10^7 CFU/mL of four wild strains of *Campylobacter jejuni* (Mean \pm SEM).



a, b, c, d Means within trials with no common superscripts differ significantly ($P < 0.05$). All *Campylobacter* data were Log_{10} transformed for statistical analysis ($n=10$ samples/dose).

Figure 3: The effect of different concentrations (in percentage) of BR on chicken skin samples (2 ± 0.1 g) challenged with approximately 2.4×10^7 CFU/mL of four wild strains of *Campylobacter jejuni* (Mean \pm SEM).



^{a, b, c} Means within trials with no common superscripts differ significantly ($P < 0.05$). All *Campylobacter* data were Log_{10} transformed for statistical analysis ($n = 10$ samples/dose).



Attn: University of Arkansas Graduate School

April 6, 2015

Dear Sir,

I attest that Basanta Raj Wagle was first author of the manuscript cited below and completed at least 51% of the work for the paper.

B. R. Wagle, A. M. Donoghue, K. Arsi, A. Woo-Ming, S. Shrestha, A. Upadhyay, P. J. Blore, K. Venkitanarayanan and D. J. Donoghue. Efficacy of β -resorcylic acid to reduce *Campylobacter jejuni* in pre-harvest and post-harvest poultry.

Yours Sincerely,

Dan J. Donoghue,
Professor,
Department of Poultry Science
POSC O-114
University of Arkansas
Fayetteville, AR 72701
Phone: (479) 575-2913
Email: ddonogh@uark.edu



MEMORANDUM

TO: Daniel Donoghue
FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee
DATE: September 12, 2012
SUBJECT: IACUC Protocol APPROVAL
Expiration date : **September 15, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol #13010- "**Use of natural compounds to reduce foodborne pathogens in preharvest poultry**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] prior to initiating the changes. If the study period is expected to extend beyond **09-15-2015**, you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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UNIVERSITY OF ARKANSAS

Office of Research Compliance

February 9, 2012

MEMORANDUM

TO: Dr. Dan Donoghue

FROM: W. Roy Penney
Institutional Biosafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 06021

Protocol Title: "Reducing Food Borne Pathogens in Poultry"

Approved Project Period: Start Date: February 14, 2012
Expiration Date: February 13, 2015

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 06021, "Reducing Food Borne Pathogens in Poultry". You may continue your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

CONCLUSION

Campylobacteriosis is one of the leading foodborne illnesses in United States, and is associated with the consumption of poultry and poultry products. Reducing *Campylobacter* in these species will reduce the burden of this disease. Unfortunately, most strategies employed to reduce *Campylobacter* in chicken have either not been successful or produced inconsistent results. One potential control strategy is the use of β -resorcylic acid (BR), a phytophenolic compound classified by the US FDA as “Everything Added to Food in the United States” (EAF 3045) and is therefore deemed safe for consumption. This compounds has antibacterial activity against *Salmonella*, however, its efficacy to control *Campylobacter* in poultry has not been evaluated. Preliminary studies in our laboratory demonstrated that BR kills *Campylobacter jejuni*, *in vitro*. Therefore, the objective of this study was to evaluate whether BR would reduce *Campylobacter* in chickens. In pre-harvest studies, day of hatch chicks were fed one of five treatments (0, 0.25, 0.5, 1 or 2%BR) in the first trial, whereas a second trial was conducted including two additional doses of 0.75% & 1.5% BR (n=10 chicks/dose). Birds were challenged with mixture of four wild strains of *C. jejuni* ($\sim 10^6$ CFU/mL) on day 7 and cecal samples were collected on day 14 and enumerated for *Campylobacter*. In post-harvest studies four trials, two each on thigh skin and breast meat, were conducted. Chicken skin or meat samples (2 ± 0.1 g) were inoculated with 50 μ L of *C. jejuni* ($\sim 10^7$ CFU/mL). Following 30 min of attachment, samples were dipped into their respective treatment solutions (0, 0.5, 1, 2% BR) for 30 s and suspended 2 min (n=10 samples/dose) and evaluated for reduction in *Campylobacter* counts. Data were analyzed by PROC GLM procedure of SAS ($P < 0.05$). *Campylobacter* counts were reduced by 1.4 Log CFU/g for the 2% dose in the first pre-harvest trial and by 4.2 or 2.8 Log CFU/g for the 0.5 % & 1% BR doses in the second pre-harvest trial. In the post-harvest studies,

all doses of BR significantly reduced *Campylobacter* counts in both meat and skin. Results of these experiments suggest post-harvest application of BR is the most effective treatment and may help reduce the incidence of human campylobacteriosis.